Acclimatization of 'VR043-43' (*Vitis vinifera* x *Vitis rotundifolia*) grapevine rootstock

Marília Pereira Machado[1]; Luiz Antonio Biasi[2]*; Marlice Ritter[3]; Luciana Lopes Fortes Ribas[4]; Flávio Zanette[2]

[1]*UFPR – Programa de Pós-Graduação em Agronomia.*
[2]*UFPR – Depto. de Fitotecnia e Fitossanitarismo, R. dos Funcionários, 1540, C.P. 19061 – 81531-990 – Curitiba, PR – Brasil.*
[3]*UFPR – Laboratório de Micropropagação de Plantas.*
[4]*UFPR – Depto. de Botânica, Setor de Ciências Biológicas, C.P. 19031 – 81531-990 – Curitiba, PR – Brasil.*
**Corresponding author <biasi@ufpr.br>*

ABSTRACT: The pre-acclimatization stage can be used to improve micropropagation protocols and increase the yield of produced plants. The influence of sucrose and photon flux density (PFD) levels on the acclimatization of *in vitro*-grown 'VR043-43' (*Vitis vinifera* x *Vitis rotundifolia*) grapevine rootstocks was evaluated. Rooted shoots were obtained from 4-week-old *in vitro* shoots cultivated in QL (Quoirin and Lepoivre, 1977) culture medium supplemented with 15, 30 and 45 g L^{-1} of sucrose. The experiment was kept in a 25 ± 2°C growth room, under 16-h photoperiod and PFD of 18 µmol m^{-2} s^{-1} or 43 µmol m^{-2} s^{-1}. Plants were transferred to an intermittent misting system greenhouse for 10 d followed by 20 d of once-a-day watering routine using a hand-held hose. Plant height was influenced by sucrose concentration, and shoots produced on media supplemented with 30 g L^{-1} sucrose were the tallest (5.0 cm). The largest leaf area was obtained with 31.3 g L^{-1} of sucrose, under the PFD of 43 µmol m^{-2} s^{-1} (13.3 cm^2). Absence of sucrose in the culture medium led to a significant reduction in leaf area at both PFDs. Shoot (aerial part) dry matter was largest when 30 or 45 g L^{-1} of sucrose (17.5 and 16.7 mg per plant, respectively) were used. Microcuttings rooted in all sucrose concentrations tested. The highest survival percentage (100%) during *ex vitro* acclimatization was obtained for shoots cultured in media supplemented with 45 g L^{-1} of sucrose under both PFDs tested.
Key words: tissue culture, sucrose, micropropagation

Aclimatização do porta-enxerto de videira 'VR043-43' (*Vitis vinifera* x *Vitis rotundifolia*)

RESUMO: A fase de pré-aclimatização pode ser utilizada para aperfeiçoar os protocolos de micropropagação e aumentar o rendimento na produção de mudas. Avaliou-se a influência da sacarose e níveis de densidade de fluxo de fóton (DFF) *in vitro*, na sobrevivência das mudas do porta-enxerto de videira 'VR043-43'(*Vitis vinifera* x *Vitis rotundifolia*), na fase de aclimatização. Microestacas obtidas de brotações *in vitro* foram cultivadas em meio de cultura QL suplementado 15, 30 e 45 g L^{-1} de sacarose. O experimento foi mantido em sala climatizada com temperatura de 25 ± 2°C, fotoperíodo de 16 horas e DFF de 18 µmol m^{-2} s^{-1} ou 43 µmol m^{-2} s^{-1}. As plantas foram transferidas para uma câmara de nebulização intermitente por 10 d e mantidas durante 20 d em casa de vegetação com irrigação manual. A altura das plantas foi influenciada pelas concentrações de sacarose, sendo a maior altura (5,0 cm) obtida com a concentração de 30 g L^{-1} de sacarose. A maior área foliar foi obtida com 31,3 g L^{-1} de sacarose na DFF de µmol m^{-2} s^{-1} (13,3 cm^2). A ausência de sacarose no meio de cultura promoveu redução significativa na área foliar nas duas DFFs testadas. A matéria seca da parte aérea foi maior quando o meio de cultura foi suplementado com 30 ou 45 g L^{-1} de sacarose (17,5 e 16,7 mg por planta, respectivamente). Houve enraizamento das microestacas em todas as concentrações de sacarose testadas. Alta porcentagem de sobrevivência (100%) durante a aclimatização *ex vitro* foi obtida quando as brotações foram cultivadas no meio de cultura suplementado com 45 g L^{-1} de sacarose em ambas DFFs testadas.
Palavras-chave: cultura de tecidos, sacarose, micropropagação

Introduction

A pre-acclimatization stage, which applies techniques to make the *in vitro* environment more similar to the natural one, is suggested to enhance survival of micropropagated plants under *ex vitro* conditions (George, 1993). The pre-acclimatization stage can be used to improve micropropagation protocols and, consequently, to increase the yield of produced plants (Hoffmann et al., 2001).

The presence of sugar in the culture medium inhibits photoautotrophic capacity of plants, causing reduced growth and death of plants later in the acclimatization process (Kozai, 1991). In addition, presence of sucrose for a prolonged period in the culture medium may also cause loss of chlorophyll synthesizing capacity by the

cells (George, 1993). However, the presence of a carbohydrate source is essential for *in vitro* rooting of many species and determinant for transplantation success (Srikandarajah and Mullins, 1981; Grattapaglia and Machado, 1998). Furthermore, *in vitro* root development usually enhances transplanting success because functioning roots can create a favorable plant water balance (Diaz-Perez et al., 1995).

Gribaudo and Fronda (1993) suggested a reduced sucrose concentration in the culture medium in the phase preceding the transfer of shoots or plants to *ex vitro* environment. This is done in order to enhance photosynthetic capacity and adaptation of plants to autotrophic conditions. Furthermore, Gribaudo and Fronda (1993) affirmed that an increase in light intensity facilitates the acclimation process since it promotes photosynthesis, improves water relationship between the tissues of the plant and external environment and thickens epicuticular wax in the leaves surface. However, PFD necessary for the *in vitro* cultivation of organs and tissues may differ depending of the explant type, the plant species and the micropropagation stage (Economou and Read, 1987).

The objective of this paper was to evaluate the influence of sucrose and PFD on the survival of *in vitro* produced 'VR043-43' grapevine rootstock plants during acclimatization.

Material and Methods

One-node stem pieces excised from *in vitro* 'VR043-43' grapevine rootstock shoots, which had been previously subcultured five times, were placed in test tubes (25 mm Ø x 150 mm high) containing 10 mL of QL medium (Quoirin and Lepoivre, 1977) with MS vitamins (Murashige and Skoog, 1962). This medium was supplemented with 100 mg L^{-1} of myo-inositol, 6 g L^{-1} of agar, and the following sucrose concentrations: 0, 15, 30 and 45 g L^{-1}. No growth regulator was added. Media was sterilized by autoclaving at 121°C, for 20 minutes at 1 atm.

The tubes were capped with aluminum foil and sealed with plastic film and kept in an acclimatized room at 25 ± 2°C with a 16-h photoperiod provided by cool light fluorescent lamps, and PFD of 18 μmol m^{-2} s^{-1} or 43 μmol m^{-2} s^{-1} for four weeks.

Transplantation of the tissue-cultured plantlets to an intermittent mist system greenhouse was done after four weeks of *in vitro* culturing. For that, culture tubes were placed inside this environment two days before the transplant. After that, the roots of the plants were washed in order to remove the agar and transferred to 53 cm^3 tubes, containing a commercial substrate.

Plants were kept under the intermittent mist system for ten days subjected to the following watering regime: 15 s watering every 30 min from 8:00. to 17h00; 15 s watering every hour from 17h00 to 23h00; and 15 s watering every three hours from 23h00 to 8h00. After that, plants remained inside the greenhouse for 20 d, under daily irrigation using a hand-held hose.

Two evaluations were performed: one after removing the plantlets from the tubes to the greenhouse and the other after 30 d inside the greenhouse. The variables analyzed were height, number of leaves, percentage of roots, shoot (aerial part) dry matter, root dry matter and the leaf area. The latest was determined by using a Win Rhizo LA 1600 (Regent Instruments Inc.) with 100 Wn resolution.

A 2 × 4 factorial (two PFDs and four sucrose concentrations) with four replicates of 40 explants per treatment in a randomized complete block design was used. The experiment was repeated once. Results were analyzed using analysis of variance (ANOVA), and means were compared at the 5% level of probability according to Duncan's multiple range test using MSTATC (Michigan State University) program. For some results, a second-degree polynomial model was fitted. The adjusted equation model was: $Y = b_0 + b_1X + b_2X^2$ where Y was the variable, X the applied treatment, and b_0, b_1, b_2 the coefficients of the model.

Results and Discussion

Plant height was positively affected by sucrose concentration up to 30.1 g L^{-1} (Figure 1). However, greater growth responses were observed for 41B grapevine rootstock (*Vitis vinifera 'Chasselas'* x *Vitis berlandieri*) aerial parts when 25.0 or 37.5 g L^{-1} of sucrose were added to the culture medium (Fila et al., 2008). For the du Lot and Chardonnay grape cultivars, as well as to 'Gravesac' grapevine rootstocks, plant height was significantly superior when medium was supplemented with sucrose compared to a sucrose-free medium (Galzy and Compan, 1992; Silva and Doazan, 1995).

Sucrose concentration and PFD caused an effect on the number of leaves per plant, and the interaction between both factors was detected by the analysis of variance. The number of leaves increased up to 34.2 g L^{-1} of sucrose (5.7 leaves), under 18 μmol m^{-2} s^{-1}. At 43 μmol m^{-2} s^{-1}, the maximum point on the curve (6.9 leaves) corresponded to the 29 g L^{-1} sucrose concentration. Higher PFD not only promoted more leaves per plant but also less sucrose was necessary to reach its maximum point

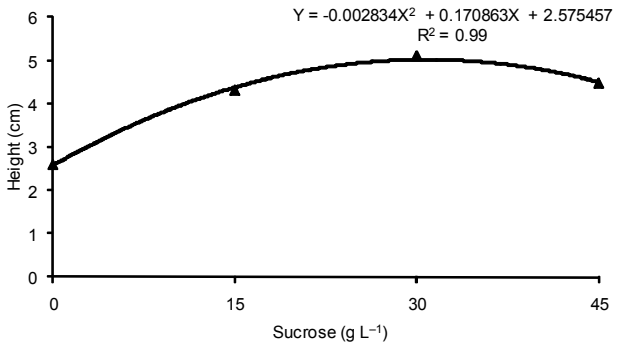

Figure 1 – Effect of sucrose concentrations on height of micropropagated 'VR043-43' grapevine rootstock plants.

(Figure 2). Number of leaves per plant of *Limonium* 'Misty Blue' was also greater under more elevated photon flux densities when combined to 30 g L^{-1} of sucrose (Lian et al., 2002). Contrasting results were obtained by Mosaleeyanon et al. (2004) who obtained greater leaf numbers per plant of *Samanea saman* when using sucrose-free culture media.

The largest leaf area, estimated by regression equation, was obtained with 31.3 g L^{-1} of sucrose, under 43 μmol m^{-2} s^{-1} (13.3 cm^2) (Figure 3). Borghezan et al. (2003) observed a 12.5 ± 1.3 cm^2 leaf area in 'VR043-43' rootstock grapevines when using the culture medium DSD1 (Silva and Doazan, 1995) with 20 g L^{-1} of sucrose under 40 to 45 μmol m^{-2} s^{-1}.

Under the lowest PFD tested (18 μmol m^{-2} s^{-1}), a higher sucrose concentration (34.2 g L^{-1}) was necessary to reach the highest point of the regression curve (5.7 cm^2) (Figure 3).This value was inferior to the one obtained under the highest photon flux density. However, no difference in leaf area was observed under three PFDs (35, 60 and 85 μmol m^{-2} s^{-1}) (Viña et al., 2001) in *Persea Americana,* for instance. Herein, absence of sucrose in the culture medium reduced the leaf area of plants under both PFDs tested (Figure 3). Leaf area of *Nicotiana tabacum* plants was also lower in the absence of sucrose compared to the medium supplemented with 30 g L^{-1} of sucrose (Kdlećek et al., 2001).

The analysis of variance confirmed the influence of both factors (sucrose concentration and PFD) on the shoot dry matter production, but no interaction between the two studied factors was detected. The best results in shoot dry matter production were obtained with 30 and 45 g L^{-1} of sucrose (17.5 and 16.7 mg per plant, respectively) (Figure 4). Such results suggest that sucrose, besides interfering in the root system of *in vitro* plants, plays an important role in the aerial part formation of the micropropagated plants. For the PFD levels tested (18 and 43 μmol m^{-2} s^{-1}), no difference between dry matter averages (11.9 mg per plant and 13.6 mg per plant, respectively) was obtained. However, Tichá et al. (1998) observed a positive effect in biomass and leaf area of *Nicotiana tabacum* plants with addition of 30 g L^{-1} sucrose in the culture medium and Galzy and Compan (1992) obtained similar results with *Vitis rupestris*.

The smallest dry matter of the aerial part (5.0 mg per plant) was obtained when sucrose was absent from the culture medium, probably due the necessity of increasing not only the PFD but *in vitro* CO_2 enrichment and humidity in the culture tubes environment in order to promote photosynthesis, transpiration and accumulation of dry matter (Kozai and Nguyen, 2003). This is due to the increase CO_2 concentration promotes increase photosynthesis, due to its direct effect on enzyme RUBISCO (ribulose–1.5-biphosphate carboxylase), and the decrease in relative humidity with greater gas exchanges in the tube increases significantly the rate of transpiration, and consequently the absorption of water and nutrients. According to Yoon et al. (2009), *in vitro* CO_2 enrichment and sugar deprivation promoted most of the growth parameters during *in vitro* growth and *ex vitro* acclimatization of *Phalaenopsis* plantlets.

Rooting occurred regardless the sucrose concentration tested, being the interaction between sucrose concentration and PFD to the rooting percentage, according to the analysis of variance (Figure 5).

The highest rooting percentage (95%) was obtained when sucrose was absent from the culture medium, allied to PFD of 18 μmol m^{-2} s^{-1} (Figure 5A). The presence

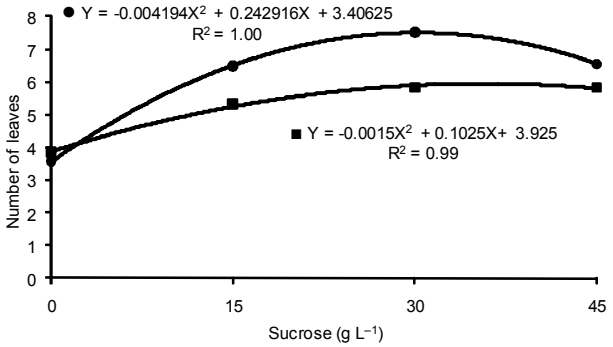

Figure 2 – Effect of sucrose concentrations and photon flux densities (■) 18 μmol m^{-2} s^{-1} and (●) 43 μmol m^{-2} s^{-1} on leaf number of micropropagated 'VR043-43' grapevine rootstock plants.

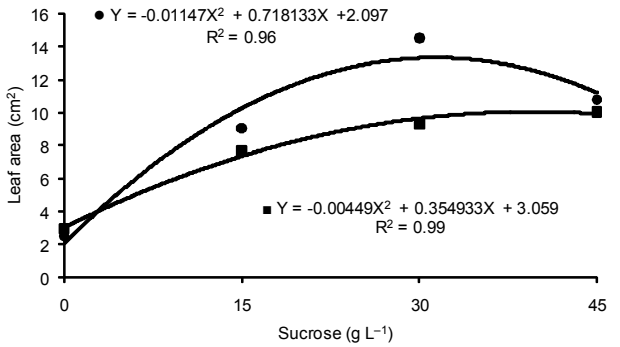

Figure 3 – Effect of sucrose concentrations and photon flux densities (■) 18 μmol m^{-2} s^{-1} and (●) 43 μmol m^{-2} s^{-1} on the leaf area of micropropagated plants of 'VR043-43' grapevine rootstocks.

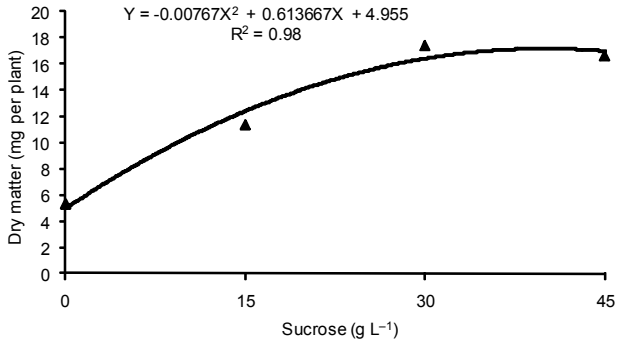

Figure 4 – Effect of sucrose concentrations on dry matter production of aerial parts of micropropagated 'VR043-43' grapevine rootstock plants.

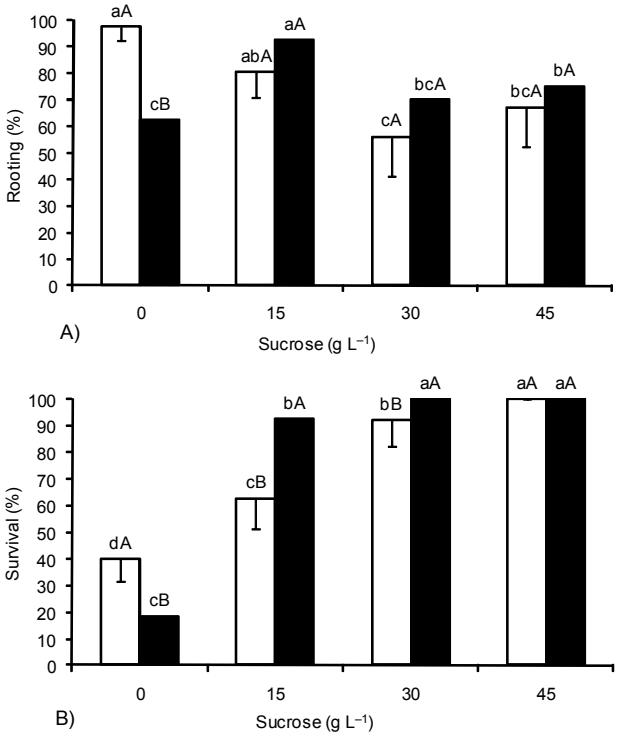

Figure 5 – Effect of sucrose concentrations and photon flux densities (□) 18 µmol m^{-2} s^{-1} and (■) 43 µmol m^{-2} s^{-1} on; A) *in vitro* rooting of shoots and B) survival after acclimatization of micropropagated 'VR043-43' grapevine rootstock plants. Letters lowercase (sucrose concentrations) or uppercase (photon flux densities) above bars indicate differences (Duncan's multiple range test). Error bars represent ± S.E. for means.

of sucrose was fundamental for the development of *in vitro* roots of strawberry plants, and no rooting response was obtained in its absence (Calvete et al., 2002). However, a 60% increase in rooting of M-26 apple tree rootstocks was obtained when sucrose was reduced from 30 to 10 g L^{-1} (Simmonds, 1983).

PFD influences shoot growth and proliferation and may directly affect formation of roots, which may be reduced in excessive irradiance (Economou and Read, 1987). Herein, there was no negative effect exerted by the higher PFD tested on rooting percentage of the rootstock plantlets. A reduction in rooting percentage was only observed if sucrose was lacking in the culture medium (Figure 5A).

ANOVA confirmed an effect of both factors (sucrose concentration and PFD) and interaction between them on the survival percentage of acclimatized plants. The highest surviving percentage of plantlets (100%) during *ex vitro* acclimatization was obtained if shoots were cultured in media containing 45 g L^{-1} of sucrose, under both PFDs and with the concentration of 30 g L^{-1} if combined to PFD of 43 µmol m^{-2} s^{-1} (Figure 5B). Concentrations between 30 and 45 g L^{-1} during pre-acclimatization were beneficial to survival of seedlings in later transplantation to greenhouse (Riquelme et al., 1991). *In vitro* culture of *Nicotiana tabacum* cv. Samsun shoots in medium containing sucrose, under elevated PFD, was important for the further *ex vitro* growth of micropropagated plants (Kdleček et al., 2001).

Absence of sucrose in the culture medium resulted in low plant survival, mainly under the PFD of 43 µmol m^{-2} s^{-1} where only 18% of the plants survived (Figure 5B). On the other hand, plants grown under photomixotrophic or heterotrophic *in vitro* conditions, in a medium containing sucrose, have low photosynthetic ability (Mosaleeyanon et al., 2004). These authors suggest that this may be the cause for the reduced growth and low percentage of plant survival after transference to *ex vitro* environment. However, they also recognized that for some species or phases of *in vitro* cultivation, sugar supplementation might be beneficial. A reduction in sucrose concentration in the rooting medium and an increase in PFD favored both photoautotrophic and vegetative stages of *Vitis* plantlets, contributing to their survival and accelerating their growth after transferring to *ex vitro* environment (Reuther, 1991).

The results obtained herein agree with Lees (1994), who concluded that the effects of light associated with the presence of a carbohydrate in the culture medium affect the efficiency of the micropropagation process. Lack of sucrose in the culture medium and higher luminous intensity impaired the *in vitro* performance and *ex vitro* acclimatization of the plants. Thus, the sucrose is necessary to meet the metabolic needs of the explants.

Conclusions

Supplementation of sucrose to the culture medium, under the PFD of 43 µmol m^{-2} s^{-1} promoted better growth and development of *in vitro* 'VR043-43' grapevine rootstock cuttings. The higher survival in the *ex vitro* environment corresponded to the higher sucrose concentration in the culture medium.

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